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Lipase-Catalyzed Esterification of Selected Phenolic Acids With Linolenyl Alcohols in Organic Solvent Media

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Abstract

Lipase-catalyzed esterification of selected phenolic acids with linolenyl alcohols was investigated in selected organic solvent media. The enzyme activity for the esterification of dihydrocaffeic acid with linolenyl alcohol in solvent mixtures of hexane/2-butanone of 75:25 (v/v) and 65:35 (v/v) was 0.88 and 0.47 μmol of esterified dihydrocaffeic acid/(g of solid enzyme·min), respectively, with a corresponding esterification yield of 76 and 58%, respectively. However, the esterification of ferulic acid with linolenyl alcohol in the reaction medium of hexane/2-butanone of 65:35 (v/v) resulted in a low yield (16%). Using the reaction medium of hexane/2-butanone of 75:25 (v/v), an increase in linolenyl alcohol concentration with a concomitant use of a constant amount of dihydrocaffeic acid resulted in an increase in esterification yield. The highest esterification yield of 99% was obtained with a ratio of dihydrocaffeic acid to linolenyl alcohol of 1:8 after 7 d of reaction. Biosynthesis of the end product, linolenyl dihydrocaffeate, was confirmed by electrospray ionization mass spectroscopy structural analysis; the ester product demonstrated an antiradical activity close to that of α-tocopherol.

Index Entries: Lipase; esterification; organic media; linolenyl alcohol; phenolic acids.

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Introduction

In recent years, there has been an increasing interest in the development of naturally occurring antioxidants, which are presumed safe compared with synthetic ones *(1)*. In addition to many of their vital proprieties in biologic systems such as anti-inflammatory and anticarcinogenic activities *(2,3)*, most phenolic acids as well as their derivatives are known to be potent antioxidants *(4)*. However, the hydrophilic property of phenolic acids has been reported as a serious disadvantage, hence limiting their effectiveness as antioxidants in fat and oil systems *(5)*. To overcome this limitation, the addition of aliphatic side-chain groups to phenolic acids via esterification has been investigated *(5,6)*. Such modifications could lead to the production of valuable phenolic lipid esters with potential emulsifying and antioxidant properties *(7,8)*. The fatty alcohol esters of ferulic acid have been found naturally in the stem bark of *Pavetta owariensis*, an anthelmintic plant *(9)*, as well as in honeybee propolis, a product widely used in folk medicine *(10,11)*.

Lipase-catalyzed biosynthesis of phenolic lipid esters in organic media has been successfully used as an alternative to the conventional chemical processes, which do not meet the necessary requirement for food applications *(12,13)*. However, most of the research reported in the literature is aimed at the esterification of phenolic acids with short- or medium-chain fatty alcohols *(5,6,13)*; there are only a few reports on the esterification of phenolic acids with long-chain unsaturated fatty alcohols that could offer numerous health benefits *(14,15)*.

The present work is part of ongoing research in our laboratory *(16,17)* aimed at the development of a biocatalysis process for the production of selected phenolic lipid esters with nutritional and functional properties. The specific objectives of this study were to investigate the esterification of selected phenolic acids, including dihydrocaffeic and ferulic acids, with linolenyl alcohol, using Novozym 435 in organic solvent media and to determine the optimal acid/alcohol molar ratio as well as the radical scavenging activity of the potential products.

Materials and Methods

Chemicals

Commercial immobilized lipase from *Candida antarctica* (Novozym 435, with an activity of 10,000 propyl laurate units per g of solid enzyme) was obtained from Novozymes Nordisk A/S (Bagsværd, Denmark). Ferulic and dihydrocaffeic acids as well as 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) were purchased from Sigma (St. Louis, MO), and linolenyl alcohol was obtained from Nuchek Prep (Elysian, MN). Organic solvents of analytical and high-performance liquid chromatography (HPLC) grades were purchased from Fisher (Fair Lawn, NJ).

Enzymatic Esterification

Lipase-catalyzed esterification of dihydrocaffeic acid or ferulic acid with linolenyl alcohol was carried out in 50-mL Erlenmeyer flasks according to the method developed previously in our laboratory *(17)*. The reaction mixture was composed of 10 m*M* fatty alcohol and phenolic acid in 9 mL of hexane/2-butanone mixtures of 75:25 (v/v) or 65:35 (v/v). The esterification reaction was initiated by the addition of 25 mg of solid Novozym 435. The Erlenmeyer flasks were incubated under vacuum at 55°C with continuous shaking at 150 rpm in an orbital shaker (New Brunswick Scientific, Edison, NJ). Control trials, without enzyme, were carried out in tandem with enzymatic reactions. Enzymatic reactions were halted by removing the immobilized lipase by decantation. The effect of various molar ratios of dihydrocaffeic acid to linolenyl alcohol of 1:1, 1:2, 1:4, and 1:8 on the esterification yield was investigated using a constant concentration of 10 m*M* dihydrocaffeic acid.

HPLC Analysis

The esterification reaction was monitored by HPLC analysis according to a method reported previously by Sabally et al. *(17)*. Sample amounts of 0.3 mL were dried down under a gentle stream of nitrogen, and the resultant mixtures were resolubilized in 1.2 mL of acetonitrile before HPLC analysis using a Beckman Gold System (Model 126; Beckman, San Ramon, CA). The reaction components were separated on a Zorbax SB-C18 reverse-phase column (250 × 4.6 mm, 5 µm; Agilent, Wilmington, DE). Elution was carried out with a gradient of acetonitrile and deionized H_2O. The elution, with a flow rate of 1 mL/min, was initiated at an acetonitrile:H_2O ratio of 85:15 (v/v) and maintained for 5 min before reaching 100% acetonitrile within an additional 15 min, which was maintained further for 5 min. Reaction components were detected at 205 and 280 nm. A calibration curve was constructed using different concentrations of dihyrocaffeic acid.

Esterification yield was defined as the concentration of esterified dihydrocaffeic acid divided by its initial concentration, then multiplied by 100. Enzyme activity was calculated from the slope of the linear portion of the plot of esterified dihydrocaffeic acid vs reaction time and expressed as micromoles of esterified dihydrocaffeic acid per gram of solid enzyme per minute.

Determination of Water Activity

The water activity (a_w) of the reaction mixtures was measured using a Novosina AW SPRINT TH-500 System (Axair, Pfaffikon, Switzerland) calibrated with saturated salt solutions of LiCl, $MgCl_2$, $Mg(NO_3)_2$, NaCl, $BaCl_2$, and $K_2Cr_2O_7$ with an a_w of 0.11, 0.33, 0.53, 0.75, 0.90, and 0.98, respectively.

Electrospray Ionization-Mass Spectrometry Analysis

Electrospray ionization-mass spectrometry (ESI-MS) was used to confirm the structure of the ester product from the esterification reaction of

dihydrocaffeic acid and linolenyl alcohol. The ester was recovered by preparative HPLC according to the method described previously and evaporated to dryness under a flow of nitrogen. The recovered fraction was reconstituted in methanolic solution of 0.5 m*M* ammonium sulfate. The sample was introduced into the electrospray ion source of a triple quadruple mass spectrometer (SCIEX API III Biomolecule mass analyzer; Thornhill, Ontario, Canada) using a syringe pump (Harvard Apparatus Model 22; South Natick, MA) at a flow rate of 1.5 mL/min. The ion spray voltage was set at 5.5 kV, and the orifice potential at 50 V.

Determination of Radical Scavenging Activity

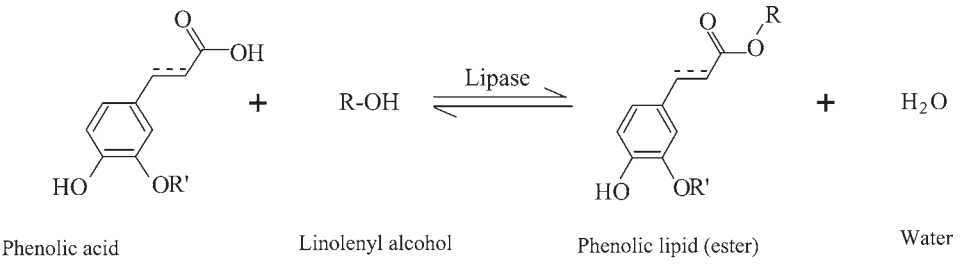
The free radical DPPH· was used to measure the scavenging activity of samples according to the method of Silva et al. *(4)*. In a 1-mL spectrophotometric cuvet, the sample was mixed with an ethanolic DPPH· solution to provide a final concentration of 20 μ*M* and 10^{-4} *M*, respectively. The reduction of DPPH· was followed spectrometrically by the decrease in absorbance at 517 nm over a period of 20 min against a blank assay without DPPH·, using a Beckman spectrophotometer (Model 650; Fullerton, CA). All assays were conducted in duplicate. The percentage of remaining DPPH· was determined as the absorbance of the remaining DPPH· at any given time (minutes) divided by the absorbance of DPPH· used as a control trial multiplied by 100.

Results and Discussion

Lipase-Catalyzed Esterification of Phenolic Acids With Linolenyl Alcohol

Lipase-catalyzed esterification of dihydrocaffeic acid or ferulic acid with linolenyl alcohol (Fig. 1) was performed in selected organic solvent media using Novozym 435 as biocatalyst. One of the main limitations in the biosynthesis of phenolic lipid esters has been the low solubility of hydrophilic phenolic acids in the hydrophobic reaction media *(5)*. In our previous work *(17)*, a binary solvent mixture of hexane and 2-butanone at a ratio of 75:25 (v/v) was determined to be the most appropriate reaction medium for the esterification of dihydrocaffeic acid with linoleyl alcohol. However, a higher proportion of 2-butanone in the reaction medium was required to solubilize ferulic acid compared with dihydrocaffeic acid. The experimental findings (data not shown) indicated that the mixture of hexane/2-butanone at a ratio of 65:35 (v/v) was the most appropriate reaction medium for the esterification of ferulic and with linolenyl alcohol.

Figure 2 depicts the time courses of lipase-catalyzed esterification of dihydrocaffeic acid or ferulic acid with linolenyl alcohol. Using a hexane/2-butanone mixture of 75:25 (v/v) as a reaction medium, the esterification yield of dihydrocaffeic acid with linolenyl alcohol increased drastically to 59% after 2 d of reaction, which was followed by a further slow increase,



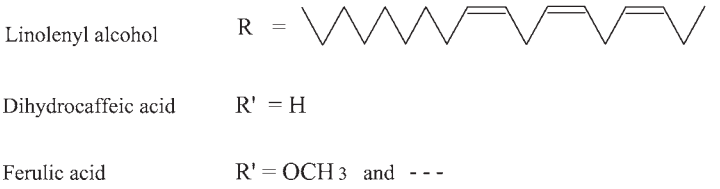


Fig. 1. Reaction scheme of lipase-catalyzed esterification of phenolic acids with linolenyl alcohol.

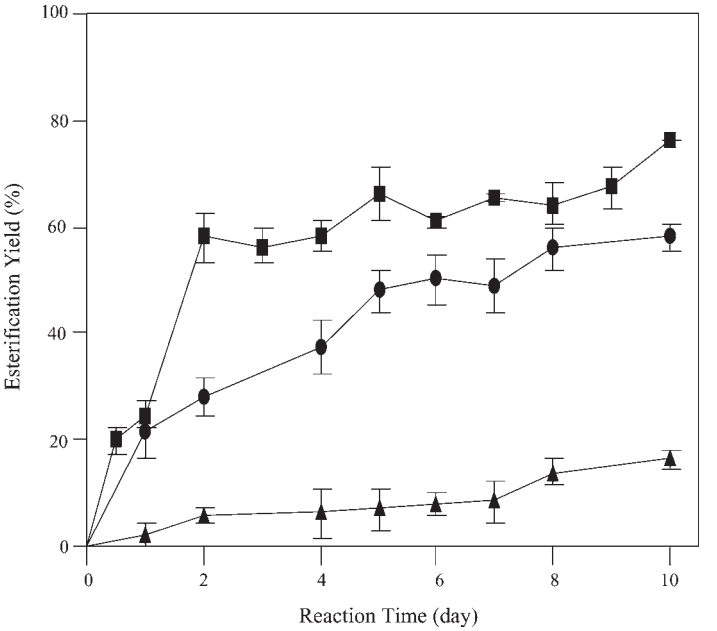


Fig. 2. Lipase-catalyzed esterification of dihydrocaffeic acid with linolenyl alcohol in hexane/2-butanone mixture of (■) 75:25 (v/v) and (●) 65:35 (v/v) as well as that of ferulic acid in same solvent mixture of (▲) 65:35 (v/v).

to reach its maximum of 76% after 10 d of reaction; however, in a hexane/2-butanone mixture of 65:35 (v/v), the extent of esterification over the time course of the reaction was low, with a maximum yield of 58% after 10 d of

Table 1
Effects of Nature of Phenolic Acid
on Lipase-Catalyzed Esterification of Linolenyl Alcohol

Phenolic acid	Enzyme activity[a]	a_w[b]	Esterification yield (%)[c]
Dihydrocaffeic[d]	0.88	0.12	76.3 (0.2)[f]
Dihydrocaffeic[e]	0.47	0.18	58.2 (3.6)[f]
Ferulic[e]	0.06	0.15	16.3 (5.8)[f]

[a]Enzyme activity was defined as the concentration in micromoles of esterified phenolic acid per gram of solid enzyme per minute.

[b]Average values of a_w calculated over the course of the reaction.

[c]The esterification yield was calculated as the maximum concentration of esterified phenolic acid divided by its initial concentration before reaction multiplied by 100.

[d]Esterification reaction was carried out in a 75:25 (v/v) hexane/2-butanone mixture.

[e]Esterification reaction was carried out in a 65:35 (v/v) hexane/2-butanone mixture.

[f]The relative standard deviation (RSD) was calculated from the SD of the samples divided by the mean of the samples analyzed multiplied by 100 and was based on duplicate samples.

reaction. The results (Table 1) also indicate that the a_w of the reaction medium, using both mixtures, remained quite constant, at a value <0.18, over the course of the esterification reaction. The decreases in enzyme activity and esterification yield (Fig. 2, Table 1) at a higher 2-butanone proportion could be attributed to its stripping effect on the water layer surrounding the enzyme, which is essential for the enzyme activity *(18,19)*. In addition, the decrease in the availability of the hydrophobic fatty alcohol to the enzyme at a higher proportion of 2-butanone could account for the low esterification yield *(20)*. The changes in enzyme conformation induced by the nature of organic solvents could also be an explanation for the low esterification yield in the polar medium *(21)*. The maximum yield of esterification of dihydrocaffeic acid with linolenyl alcohol (76%) is in the same range as that obtained by Sabally et al. *(17)* for the esterification of the same phenolic acid with linoleyl alcohol (75%). These experimental findings suggest that the presence of an additional double bond on the fatty alcohol has little effect on the esterification yield.

The results (Fig. 2, Table 1) also show that the enzyme activity and the esterification yield depend on the type of phenolic acid and its structural characteristics. In the hexane/2-butanone mixture of 65:35 (v/v), the extent of esterification yield over the time course of the reaction was lower with ferulic acid than with dihydrocaffeic acid. However, with both phenolic acids, the equilibrium reaction was reached after 8 d of reaction, with a maximum esterification yield of 16 and 58% for ferulic and dihydrocaffeic acid, respectively. Although the availability of ferulic acid to the enzyme was enhanced by the increase in the proportion of 2-butanone in the hexane medium, its esterification with linolenyl alcohol remained low. Guyot et al. *(22)* reported a low yield of 14% for the esterification of ferulic acid with oleyl alcohol by Novozym 435 in a solvent-free medium. The present experimental findings (Fig. 2, Table 1) suggest that the presence of the

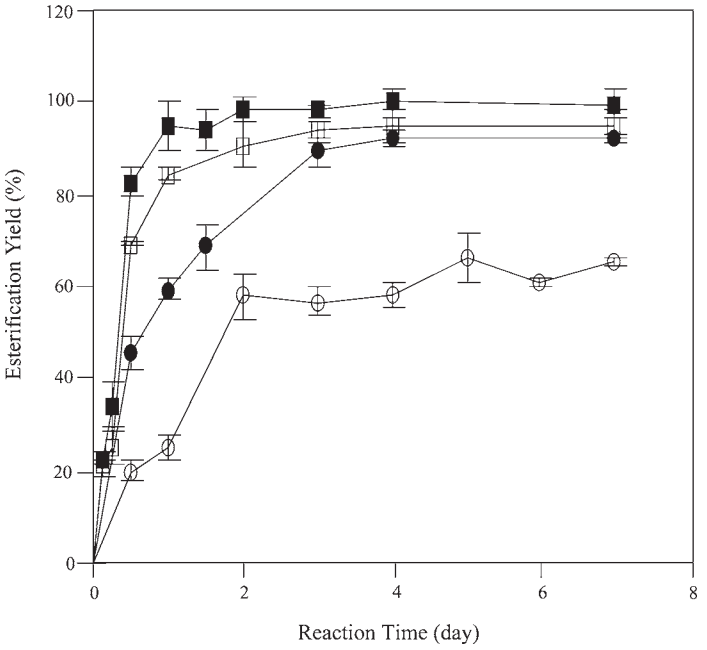


Fig. 3. Effect of dihydrocaffeic acid and linolenyl alcohol ratios of (○) 1:1, (●) 1:2, (□) 1:4, and (■) 1:8 on esterification yield in reaction medium of 75:25 (v/v) hexane/2-butanone.

methoxyl substituent and the double bond on the side chain, conjugated with the aromatic ring, on the ferulic acid could have affected its molecular binding and/or its transformation, which may have decreased the enzyme activity *(22)*. Ferulic acid was reported as a poor substrate by other research groups *(5,23)*, who attributed the low enzymatic activity to an electronic or steric phenomenon. Buisman et al. *(13)* reported that the reactivity of the carboxylic function of phenolic acids may be affected by the electron-donating substituents in their aromatic rings, hence limiting the nucleophilic attack of the alcohol.

Effect of Acid/Alcohol Ratio on Esterification Reaction

To maximize the biosynthesis of linolenyl dihydrocaffeate, the effect of substrate molar ratio on the lipase-catalyzed esterification was investigated by varying ratios of dihydrocaffeic acid to linolenyl alcohol from 1:1 to 1:8. Figure 3 presents the time courses of the esterification reaction over 7 d at different substrate molar ratios. The results indicate that decreasing the molar ratio of dihydrocaffeic acid to linolenyl alcohol resulted in a higher reaction rate and a shorter required time for reaching the reaction equilibrium. The effect of the substrate molar ratio on the reaction equilibrium time could be attributed to the difference in the solvation of the reaction components in the organic medium at different ratios *(24)*. The results also show that the maximum esterification yield increased significantly

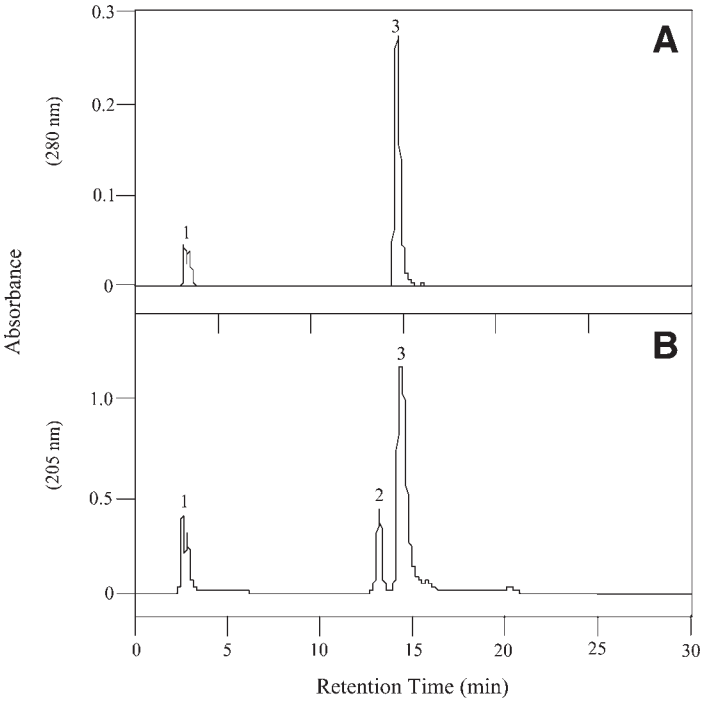


Fig. 4. Chromatogram of HPLC analysis of components of lipase-catalyzed esterification of dihydrocaffeic acid with linolenyl alcohol monitored at **(A)** 280 and **(B)** 205 nm.

from 66 to 92% when the molar ratio of dihydrocaffeic acid to linolenyl alcohol was decreased from 1:1 to 1:2; however, a further decrease in substrate molar ratio to 1:4 and 1:8 resulted in a modest increase in maximum esterification yield to 95 and 99%, respectively. Therefore, an excess of linolenyl alcohol has a beneficial effect on the esterification yield, which may be related to the substrate-partitioning effect and/or to the reaction equilibrium *(25)*. These experimental findings are in agreement with those obtained previously in our laboratory *(16,17)* in which a decrease in the substrate molar ratio, obtained by an incremental increase in the concentration of fatty alcohol, resulted in an increase in the bioconversion yield. Gutman et al. *(12)* also reported an increase in the rate of transesterifications of ethanol and *n*-butanol with benzyl esters by increasing the substrate molar ratio up to 10.

Structural Analyses of End Products

The components of the lipase-catalyzed esterification of dihydrocaffeic acid with linolenyl alcohol were subjected to HPLC analysis, using an ultraviolet/diode-array (UV/DAD) detector at 205 and 280 nm. Figure 4 shows a typical HPLC elution profile of reaction components, in which the more hydrophilic molecules were eluted prior to the hydrophobic ones. Two predominant peaks, no. 1 and 3, with a retention time of

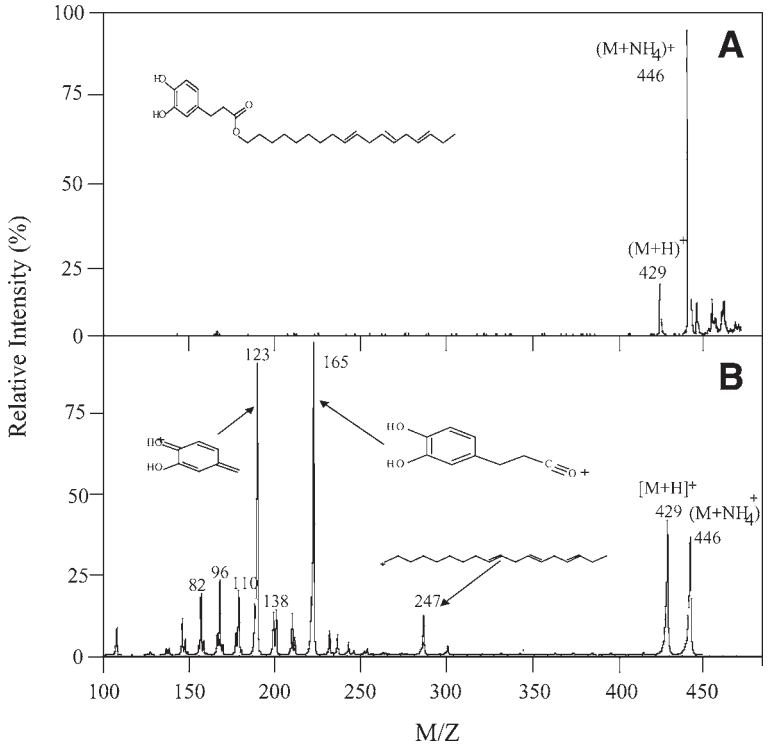


Fig. 5. ESI-MS analysis of enzymatic reaction end product at **(A)** low ionization and **(B)** collision-induced dissociation of ammoniated product.

3.0 and 15.0 min, respectively, were obtained with the elution profile at 280 nm (Fig. 4A). An additional peak, no. 2, with a retention time of 14 min, was detected at 205 nm (Fig. 4B). On the basis of retention time and UV spectral scanning of their respective standards, peaks no. 1 and 2 were determined to be the dihydrocaffeic acid and linolenyl alcohol, respectively. Peak no. 3 was characterized to be the end product, because it absorbed at both 205 and 280 nm and it was not detected in the elution profile of the blank. The results also show that the linolenyl alcohol and the end product displayed a close hydrophobic property, as demonstrated by their corresponding retention times.

The end product (peak no. 3) was recovered by preparative HPLC and subjected to ESI-MS analysis for its structural characterization. The result of the ESI-MS, at low energy bombardment (Fig. 5A), showed ion peaks at m/z 429 and 446, which represent the ionized $(M+H)^+$ and ammoniated $(M+NH_4)^+$ end products, respectively. The fragmentation pattern, obtained from the collision-induced dissociation of ammoniated product (Fig. 5B), confirmed the molecular ion species of the ester product with a mol wt of 428 g/mol.

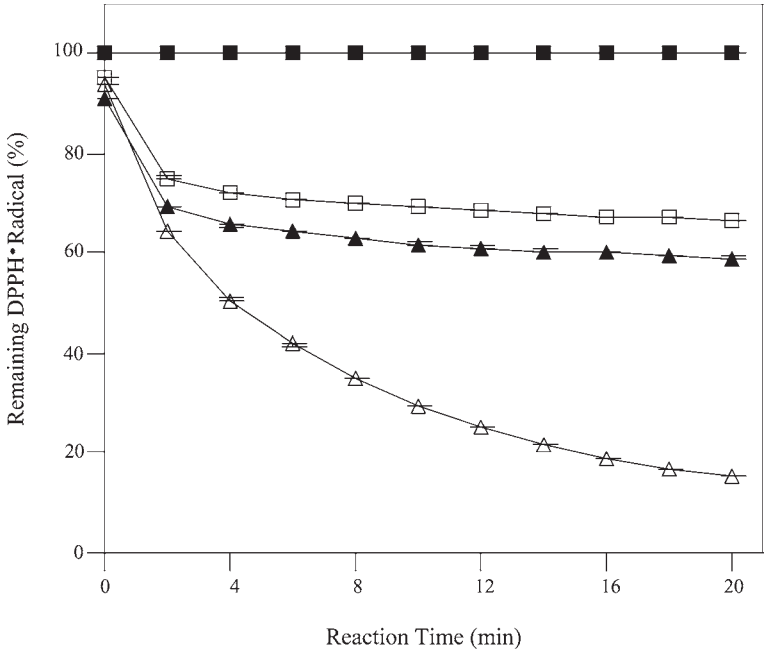


Fig. 6. Time course for DPPH· scavenging by (△) dihydrocaffeic acid and its ester with (□) linolenyl alcohol, (▲) α-tocopherol, and (■) DPPH· as control trial.

Free-Radical Scavenging Activity of Dihydrocaffeic Acid and Its Linolenyl Alcohol Ester

The radical scavenging activity of dihydrocaffeic acid and its phenolic lipid ester toward a stable free-radical DPPH· was evaluated and compared with that of α-tocopherol, a known natural antioxidant (Fig. 6). The results show that the steady state of the reaction was reached within 20 min of incubation. Dihydrocaffeic acid demonstrated the highest antiradical activity, with 83% of radical DPPH· scavenging, compared with that of its ester with linolenyl alcohol (33%) and α-tocopherol (40%). These experimental findings indicate that the esterification of dihydrocaffeic acid with linolenyl alcohol resulted in a decrease in the radical scavenging activity of the phenolic acid. These results are in agreement with our earlier work *(17)*, which indicated that the linoleyl dihydrocaffeate ester has a low radical scavenging activity compared with that of its corresponding phenolic acid. The literature *(4,26)* indicates that the radical scavenging activity of phenolic compounds was dependent on their molecular structure, especially on their hydrogen-donating ability and subsequent stabilization of the formed phenoxy radical.

Conclusion

The esterification yield of dihydrocaffeic acid with linolenyl alcohol was higher than that of ferulic acid. The presence of the methoxyl substitu-

ent and the double bond on the side chain, conjugated with the aromatic ring, on the ferulic acid may have led to an inhibitory effect on enzyme activity. Structural analysis confirmed that the lipase-catalyzed esterification resulted in the biosynthesis of a phenolic lipid molecule, linolenyl dihydrocaffeate. The free-radical scavenging activity of linolenyl dihydrocaffeate was less than that of its phenolic acid, but almost comparable with that of α-tocopherol.

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